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# Effect of Water Pollutants and Other Chemicals upon Ribonuclease Activity In vitro

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16. ABSTRACT

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# Effect of Water Pollutants and Other Chemicals upon Ribonuclease Activity *in Vitro*

G. M. CHRISTENSEN AND D. L. OLSON

*U.S. Environmental Protection Agency, Environmental Research Laboratory–Duluth, 6201 Congdon Boulevard, Duluth, Minnesota 55804*

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Ribonuclease was treated *in vitro* with 73 chemicals, many of which are environmental pollutants, including inorganic, organic, and metal–organic chemicals, pesticides and other biocides, alkyl and aryl industrial pollutants, and certain additional chemicals, to determine their effect upon enzyme activity. Palladium (II and IV) and gold (III) were the strongest inhibitors of RNase activity. Other strong inhibitors, in decreasing order of effect, were: sodium dodecyl sulfate, silver (I), EDTA, mercury (II), copper (II), thiram (fungicide), platinum (IV), malathion (pesticide), lead (II), and beryllium (II). Intermediate effects were found with other inorganic cations, many anions, and some other chemicals. A number of compounds of different chemical types caused no measurable effect. None of the chemicals tested caused a measurable activation of this enzyme.

## INTRODUCTION

The work reported here on pancreatic ribonuclease (RNase, EC 3.1.27.5) was undertaken to determine the sensitivity of an enzyme associated with genetic processes when exposed *in vitro* to various environmental pollutants and other chemicals and to point out any correlations between chemical structure (or class) and inhibitory effect. It follows earlier studies at this laboratory that were concerned with effects of environmental pollutants on selected enzymes and other biochemicals.

Much remains to be understood about the detailed mechanism of action of most toxic water pollutants on aquatic animals and about effects on other animals, including humans. General physiologic and systemic responses in test animals have been described for certain toxicants, usually after high dosages have been applied over short exposure times; but at the molecular level the sequence of significant toxicogenic events leading finally to overt disabilities is largely unknown. Enzymes no doubt are often involved in a primary way, as seems to be the case, for example, with cholinesterase inhibition by carbamate pesticides (O'Brien, 1967; Corbett, 1974). We must still determine, however, which other key enzymes respond to highly toxic chemicals and how the initial metabolic perturbations of the toxic state develop for each class of chemical.

Ribonuclease has been well characterized chemically and structurally (Josefsson and Lagerstedt, 1962; Ross *et al.*, 1962; Scheraga and Rupley, 1962; Avey *et al.*, 1967; Scheraga, 1967). Inhibition of activity has been reported by the following inorganic cations: Cu(II), Zn(II), Cd(II), Pb(II), Hg(II), Be(II), Ag(I), Mg(II), Ca(II), Mn(II), Ni(II), and Co(II) (Lamanna and Mallette, 1949; Davis and Allen, 1955; Ross *et al.*, 1962; Breslow and Girotti, 1966, 1970; Herzog and

Bigelow, 1967; Eichorn *et al.*, 1969; Jackim *et al.*, 1970; Alger, 1970). Inhibitory effects upon ribonuclease activity have also been reported for dodecyl sulfate and diethyl pyrocarbonate (Jones, 1976); phenylmercuric acetate, iodoacetate, and iodoacetamide (Zittle, 1946; Terao *et al.*, 1970); and *p*-chloromercuric benzoate (Ledoux, 1953; Dickman *et al.*, 1956).

The significance of changes in RNase activity in carcinogenesis has been discussed by Daoust and Lamirande (1975) and Liu *et al.* (1975). Since a diminution of RNase activity could be a critical event in carcinogenesis (Daoust and Lamirande, 1975), and because an increasing number of environmental pollutants are being implicated in carcinogenesis (Cairns, 1975; Fishbein, 1976; Nery 1976), we tested four carcinogens (as reported by Weisburger, 1978): methyl methanesulfonate, diepoxybutane, bis(chloromethyl) ether, and dimethylcarbamyl chloride. One consequence of RNase inhibition would be a metabolic slowdown of RNA catabolism, causing an accumulation of RNA and products such as spermine and spermidine (Maugh, 1977). In a review of RNase in normal and tumor tissue Roth (1967) stated that in certain instances RNase activity is low in tumor cells, although Chretien *et al.* (1973) reported that serum RNase activity is elevated in some tumors, and is apparently normal in other types. We need more careful evaluations of metabolic effects upon biota by environmental agents (Train, 1977), particularly those that have direct relevance to human health.

## MATERIALS AND METHODS

By following essentially the method of Kunitz (1941) and Kalnitsky *et al.* (1959), ribonuclease[1] (0.15 g) was prepared in acetate buffer (10 ml of 0.2 M, pH 5.0), as was yeast ribonucleic acid (0.7 mg/100 ml). The color reagent was 0.75% (w/v) uranyl acetate in 25% (v/v) perchloric acid.

To the enzyme preparation (50$\mu$l) was added acetate buffer (for the reference determination) or the test (inhibitor) reagent (450 $\mu$l) in acetate buffer at concentrations ranging from $2 \times 10^{-2}$ M to dilutions that had no effect upon enzyme activity. Stock solutions (0.02 M) of water-insoluble chemicals were prepared in ethanol and were then diluted with acetate buffer. If suspensions occurred, they were analyzed nonetheless. The particular solvent or solvent combination for each preparation was analyzed alone to provide the appropriate blank value. After preincubation for 15 min with mild mixing, RNA (400 $\mu$l) was added, and the reaction was allowed to proceed for 1/2 hr at 22°C with mild mixing. The precipitating agent (uranyl acetate–perchloric acid) was then added (1.00 ml), and the samples were centrifuged (3000*g*) at 1°C for 20 min. Aliquots of the supernatant (50 $\mu$l) were then withdrawn and mixed with acetate buffer (2.950 ml), and the absorbance was determined at 260 nm. Blank samples were prepared by adding the enzyme after the acidified uranyl acetate. Triplicate analyses were carried out, and the mean values were determined. The maximum activity of the enzyme preparation varied from 0.30 to 0.40 units (according to Kalnitsky *et al.*, 1959). The 73 chemicals (reagent grade) analyzed in this study are listed in Table 1.

[1] Ribonuclease-A: type I-A from bovine pancreas. Sigma Chemical Co., St. Louis, Mo. 63178. The U.S Environmental Protection Agency does not endorse any commercial product; trade names are given as an aid in identification.

TABLE 1
CHEMICALS TESTED FOR THEIR EFFECT UPON RIBONUCLEASE ACTIVITY

| Common name | Chemical description |
|---|---|
| A. Thiomides | |
| 1. Captan | *N*-(Trichloromethylthio)-4-cyclohexene-1,2-dicarboximide |
| B. Thiocarbamates | |
| 2. Thiram | Tetramethylthiuram disulfide |
| 3. Eptam | *S*-Ethyl dipropylthiocarbamate |
| C. Carbamates | |
| 4. IPC | Isopropyl-*N*-phenyl carbamate |
| 5. Carbaryl | Sevin; 1-Naphthyl-*N*-methyl carbamate |
| D. Halogenated hydrocarbons | |
| 6. DDT | Dichlorodiphenyltrichloroethane |
| 7. Lindane | 1,2,3,4,5,6-Hexachlorocyclohexane (γ-isomer) |
| 8. Dieldrin | 1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4*a*,5,6,7,8,8*a*-octahydroendo-exo-1,4,5,8-dimethanonaphthalene |
| 9. Dicofol | Kelthane; 1,1-bis[*p*-Chlorophenyl]-2,?,2-trichloroethanol |
| E. Organothiophosphates | |
| 10. Malathion | *S*[1,2-Dicarbethoxyethyl]-*O*-*O*-dimethyldithiophosphate |
| 11. Diazinon | *O*,*O*-Diethyl-*O*-(2-isopropyl-4-methyl-6-pyrimidyl) phosphorothionate |
| F. Organometals | |
| 12. Methylmercuric chloride | |
| 13. Phenylmercuric chloride | |
| G. Antibiotics | |
| 14. Chloroquine | |
| 15. Chloramphenicol | |
| 16. Neomycin | |
| 17. Sulfanilamide | |
| 18. Erythromycin | |
| 19. Penicillamine | |
| 20. Streptomycin | |
| H. Industrial chemicals | |
| 21. Aniline | |
| 22. Phenol | |
| 23. Sodium dodecyl sulfate | |
| 24. Ethanol | |
| 25. Acetone | |
| I. Inorganic salts (active cation) | |
| 26. $AgNO_3$ | |
| 27. $AlCl_3$ | |
| 28. $CuCl_2 \cdot 2H_2O$ | |
| 29. NaCl | |
| 30. $CoCl_2 \cdot 6H_2O$ | |
| 31. $HgCl_2$ | |
| 32. $ZnCl_2$ | |
| 33. $CdCl_2 \cdot 2H_2O$ | |
| 34. $BiCl_3$ | |
| 35. $FeCl_3 \cdot 6H_2O$ | |
| 36. $Pb(NO_3)_2$ | |
| 37. $NiCl_2 \cdot 6H_2O$ | |
| 38. $BaCl_2 \cdot 2H_2O$ | |
| 39. $MgCl_2 \cdot 6H_2O$ | |
| 40. $CaCl_2 \cdot 2H_2O$ | |

TABLE 1—*Continued*

| Common name | Chemical description |
|---|---|
| 41. $CrCl_3 \cdot 6H_2O$ | |
| 42. $PdCl_2$ | |
| 43. $K_2PdCl_6$ | |
| 44. $BeCl_2$ | |
| 42. $NaAuCl_4 \cdot 2H_2O$ | |
| 46. $K_2PtCl_6$ | |
| 47. $SnCl_4 \cdot 5H_2O$ | |
| 48. $MnCl_2$ | |
| J. Inorganic salts (active anion) | |
| 49. $Na_2HAsO_4$ | |
| 50. $NaAsO_2$ | |
| 51. NaCN | |
| 52. $NaN_3$ | |
| 53. $Na_2S \cdot 9H_2O$ | |
| 54. $NaIO_4$ | |
| 55. $NaBH_4$ | |
| 56. $Na_2Cr_2O_7$ | |
| 57. NaI | |
| 58. NaBr | |
| 59. NaF | |
| 60. $Na_2SeO_3$ | |
| 61. $Na_2SeO_4$ | |
| 62. $Na_2TeO_4 \cdot 2H_2O$ | |
| K. Carcinogens | |
| 63. CMEE | Chloromethyl ethyl ether (bis) |
| 64. DMCC | Dimethylcarbamoyl chloride |
| 65. MMS | Methyl methanesulfonate |
| 66. DEB | Diepoxybutane |
| L. Miscellaneous chemicals | |
| 67. MS-222 | Ethyl-*m*-aminobenzoate methanesulfonic acid |
| 68. Piperonal butoxide | 3,4-Methylenedioxy-6-propylbenzyl-*n*-butyl diethylene glycol ether |
| 69. EDTA ($2Na \cdot 2H_2O$) | Ethylenediaminetetraacetic acid |
| 70. Acetazolamide | *N*-(5-Sulfamoyl-1,3,4-thiadiazol-2-yl) acetamide |
| 71. 5-Fluorouracil | |
| 72. $H_2O_2$ | |
| 73. Warfarin | 3-(α-Acetonylbenzyl)-4-hydroxycoumarin |

## RESULTS AND DISCUSSION

The results shown in Table 2 display, for each toxicant, the molar concentration which caused a 1% inhibition of enzyme activity ($I_1$, taken to be the effect–no effect threshold) and a 50% inhibition ($I_{50}$). Both $I_1$ and $I_{50}$ values are shown in Table 2 because many chemicals produced an effect that was measurable, but not intense enough at the concentrations used to provide an $I_{50}$ value.

As seen in Table 2, the most inhibiting agents to RNase were Pd(II and IV) and Au(III). The surfactant agent sodium dodecyl sulfate was also considered highly reactive, as was Ag(I) and EDTA. The ions Hg(II), Cu(II), Pt(IV), Pb(II), and

TABLE 2
VALUES FOR $I_1$ AND $I_{50}$ (EXPRESSED AS MOLARITY OF TEST CHEMICAL) FOR *in Vitro* TESTS OF VARIOUS CHEMICALS WITH RIBONUCLEASE

| Chemical (active component) | $I_1$ | $I_{50}$ |
|---|---|---|
| Pd(II) | $3 \times 10^{-7}$ | $2 \times 10^{-6}$ |
| Au(III) | $2 \times 10^{-6}$ | $4 \times 10^{-5}$ |
| Pd(IV) | $1 \times 10^{-5}$ | $5 \times 10^{-5}$ |
| Dodecyl sulfate | $1 \times 10^{-4}$ | $3 \times 10^{-4}$ |
| Ag(I) | $1 \times 10^{-5}$ | $2 \times 10^{-3}$ |
| EDTA | $3 \times 10^{-4}$ | $2 \times 10^{-3}$ |
| Hg(II) | $1 \times 10^{-4}$ | $2 \times 10^{-3}$ |
| Cu(II) | $8 \times 10^{-4}$ | $3 \times 10^{-3}$ |
| Thiram | $1 \times 10^{-3}$ | $3 \times 10^{-3}$ |
| Pt(IV) | $1 \times 10^{-3}$ | $3 \times 10^{-3}$ |
| Malathion | $1 \times 10^{-3}$ | $3 \times 10^{-3}$ |
| Pb(II) | $1 \times 10^{-3}$ | $3 \times 10^{-3}$ |
| Be(II) | $1 \times 10^{-3}$ | $3 \times 10^{-3}$ |
| Fe(III) | $1 \times 10^{-3}$ | $4 \times 10^{-3}$ |
| Al(III) | $1 \times 10^{-3}$ | $4 \times 10^{-3}$ |
| Sn(IV) | $1 \times 10^{-3}$ | $4 \times 10^{-3}$ |
| Neomycin | $6 \times 10^{-4}$ | $4 \times 10^{-3}$ |
| Cd(II) | $5 \times 10^{-4}$ | $5 \times 10^{-3}$ |
| Ni(II) | $3 \times 10^{-4}$ | $1 \times 10^{-2}$ |
| IPC | $1 \times 10^{-4}$ | $2 \times 10^{-2}$ |
| CMEE | $1 \times 10^{-3}$ | $2 \times 10^{-2}$ |
| DMCC | $1 \times 10^{-2}$ | $4 \times 10^{-2}$ |
| MMS | $1 \times 10^{-2}$ | $6 \times 10^{-2}$ |
| Co(II) | $1 \times 10^{-4}$ | $6 \times 10^{-2}$ |
| DEB | $1 \times 10^{-2}$ | $7 \times 10^{-2}$ |
| Warfarin | $3 \times 10^{-4}$ | [a] |
| Penicillamine | $3 \times 10^{-4}$ | [a] |
| Streptomycin | $3 \times 10^{-4}$ | [a] |
| MS-222 | $5 \times 10^{-4}$ | [a] |
| $IO_4^-$ | $5 \times 10^{-4}$ | [a] |
| Diazinon | $1 \times 10^{-3}$ | [a] |
| $F^-$ | $1 \times 10^{-3}$ | [a] |

[a] Moderate effect at $10^{-2}$ M ($I_{50}$ not determinable).

[b] Slight effect at $10^{-2}$ M ($I_{50}$ not determinable): acetazolamide, DDT, piperonal butoxide, lindane, $H_2O_2$, and Bi(III).

[c] Negligible effect at $10^{-2}$ M: chloramphenicol, chloroquine, sulfanilamide, erythromycin, carbaryl, captan, eptam, dicofol, dieldrin, acetone, ethanol, aniline, phenol, 5-fluorouracil, methyl Hg(I), phenyl Hg(I), Cr(III), Mn(II), Ca(II), Ba(II), Mg(II), Zn(II), $N_3^-$, $CN^-$, $S^{2-}$, $Br^-$, $I^-$, $Cr_2O_7^{2-}$, $BH_4^-$, $SeO_4^{2-}$, $SeO_3^{2-}$, $TeO_4^{2-}$, $AsO_2^-$, $AsO_4^{3-}$, and NaCl.

Be(II) were also strongly reactive. Beryllium was in sharp contrast to other group II cations, which had no effect.

Of the 12 pesticides studied only thiram (thiocarbamate fungicide), malathion (organophosphate pesticide), and IPC (carbamate pesticide) showed significant inhibitory effects on RNase. Of the seven bacterial biocides analyzed only

neomycin caused a significant change in activity. The four structurally different organic carcinogens (methyl methanesulfonate, diepoxybutane, bis(chloromethyl) ether, and dimethylcarbamyl chloride) had a relatively low but similar effect on the enzyme. Also, low effects were found for warfarin (rodenticide), penicillamine, streptomycin, MS-222 (fish anesthetic), $IO_4^-$, diazinon (organophosphate pesticide), and $F^-$. Relatively slight effects were found for acetazolamide (intense carbonic anhydrase inhibitor), DDT, piperonal butoxide (pesticide synergist), lindane (organochlorine pesticide), $H_2O_2$, and Bi(III). The anticancer drug 5-fluorouracil had no measurable effect. The inorganic anions were considered essentially nonreactive, although there was a slight inhibitory effect from $IO_4^-$ and $F^-$. Sodium periodate may have caused an effect because of its oxidative property. A rather large number (35) of the chemicals studied had a negligible effect. Some of these compounds (i.e., $AsO_4^{3-}$, $CN^-$, $S^{2-}$, and $CH_3Hg(I)$) are relatively potent inhibitors of other enzymes, which suggests that RNase, although affected by some chemicals, can maintain full activity in the presence of other types of toxic foreign chemicals.

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